

## **Response of bacteria and phytoplankton from a subtropical front location Southern Ocean to micronutrient amendments *ex-situ***

Nagappa Ramaiah<sup>a,\*</sup>, Anand Jain<sup>a,d</sup>, Ram Murti Meena<sup>a</sup>, Ravidas K Naik<sup>c</sup>, Rishendra Verma<sup>a</sup>, Mithila Bhat<sup>b</sup>, Analia Mesquita<sup>b</sup>, Akshita Nadkarni<sup>a</sup>, Stecy Elvira D'Souza<sup>a</sup>, Tufail Ahmed<sup>a</sup>, Mandar Bandekar<sup>a</sup>, Jasmine Gomes<sup>a</sup>

<sup>a</sup>Microbial Ecology Laboratory, Biological Oceanography Division, <sup>b</sup>Chemical Oceanography Division, CSIR-National Institute of Oceanography, Dona Paula, Goa, 403004.

<sup>c</sup>Southern Ocean Group, <sup>d</sup>Arctic Group, National Center for Antarctic Ocean Research, Sada Headland, Vasco-da-Gama, Goa.

### **Abstract**

Effects of micronutrient amendments such as cobalt (Co), copper (Cu), iron (Fe) and their mixture (Co + Cu + Fe) on bacterial abundance, phytoplankton, bacterial community (BC) composition was examined during the austral summer of 2012, at a subtropical front (STF) location in the Indian Sector of the Southern Ocean (ISSO). From the 15 day long on-board experiment it became evident that there was no significant increase in total chlorophyll *a* concentration or phytoplankton cell numbers in micronutrient enriched microcosms (MEM) compared to the control microcosms (CM) with no added micronutrients. Highest bacterial abundance was observed in the Fe enriched microcosm within 5 days of incubation unlike in other microcosms. Interestingly, significant differences in the BC composition were observed between MEM and CM. In that an increase of ~ 10 unique phylotypes affiliated with *Psychrobacter* sp, *Prochlorococcus* sp, *Burkholderia* sp, *Cytophaga-flavobacteria* cluster, *Roseobacter* sp, *Bacillus* sp and *Vibrio* sp was observed in the MEM. The phylotypes affiliated with *Cyanobacteria* preferentially responded to the Co and Fe addition, while the members of *Cytophaga-Flavobacteria* cluster responded to the Cu, Fe and mixture amendments. Our results are useful to discern that availability of micronutrients, in particular of Fe, affects the bacterial abundance as well as BC composition, but not the phytoplankton growth/chlorophyll *a* concentration in the STF location. In addition, micronutrient amendments *ex-situ* appears to lead to predominance of only a few bacterial phylotypes. This shift in bacterial community composition might be due to preferential and/or versatile utilization of exogenously added micronutrients. Phylogenetic diversity of culturable bacterial populations from this sampling location was also assessed using 99 pure bacterial cultures. For this, a select set of biochemical characteristics—were examined and numerical profiling was done before subjecting to 16S rRNA sequencing based identification. This effort yielded close to 20 clusters and representative isolates from each cluster were subjected to molecular taxonomic analyses using 16S rRNA marker gene. From this analysis, ~90% of bacterial isolates were found to be affiliated with *Firmicutes* and ~10% to *Gammaproteobacteria* suggesting preponderance of only a few phylotypes in the deep chlorophyll maximum zone of this STF location.

**Keywords:** Southern Ocean, subtropical front, micronutrients, bacterial community, microcosm, DGGE

**\*Corresponding author address:** Microbial Ecology Laboratory, CSIR-National Institute of Oceanography, Dona Paula, Goa, 403004. Email: [ramaiah@nio.org](mailto:ramaiah@nio.org); Phone: (+91) 832 2450 515; Fax: (+91) 832 2450 602

## 1. Introduction

The subtropical front (STF) extends almost continuously around the Antarctic (except for the South American sector) at  $\sim 40^{\circ}\text{S}$ . It separates sub-tropical region (STR) from the subantarctic region (SAR) (Orsi et al., 1995), and strongly influences the meridional transport of heat, salt and nutrients between the Southern Ocean and subtropical gyres (Jayne and Marotzke, 2002). The subtropical frontal zone (STFZ) in the Southern Indian Ocean is enclosed by two sharp fronts: the north subtropical front (NSTF) and south subtropical front (SSTF; Belkin, 1988; Kostianoy et al., 2004). The extremely oligotrophic NSTF between  $34^{\circ}\text{S}$  and  $35^{\circ}\text{S}$  is characterized by low chlorophyll *a* concentration and high microzooplankton abundance (Jasmine et al., 2009). On the other hand, SSTF is characterized by high biological productivity (Pakhomov and McQuaid, 1996) caused by the mixing of warm macronutrient-poor but micronutrient-rich subtropical water (STW) with cool macronutrient-rich yet micronutrient-poor subantarctic surface water (SASW, Boyd et al., 1999). This makes SSTF region an important carbon sink (Currie and Hunter, 1998; Metzl et al., 1999; Metzl, 2009; Ito et al., 2010; Currie et al., 2011; Shadwick et al., 2015). Despite high primary productivity in the STF transfer of biogenic carbon to depth via biological pump is reported to be very low mainly due to efficient remineralization process and/or a high bacterial activity (Blain et al., 2002; Sedwick et al., 2002; Coppola et al., 2005). Jasmine et al (2009) observed higher abundance of microzooplankton and mesozooplankton along with higher biological productivity in the STF. Such a scenario ought to imply an active microbial loop in biogenic carbon sink in the STF region. The microbial loop is a critical part of 'biological carbon pump' and can alter the efficiency of carbon export via remineralization of organic matter back to inorganic phase and/or conversion of organic matter to biomass (Robinson and Ramaiah, 2011). Therefore, it is imperative to elucidate the bacterial diversity as well as factors influencing bacterial growth and community composition in STF region of the Indian Sector of the Southern Ocean (ISSO).

Micronutrients are essential for virtually all organisms, but are extremely scarce in the Southern Ocean (Ellwood, 2004, 2008, Ellwood et al., 2005, Croot et al., 2011, Hassler et al., 2012). Dissolved Fe concentration of 0.09 nM (Blain et al., 2002) reported from STF region is quite low. So also are ranges of dissolved-Co (24 - 49 pM; Castrillejo et al., 2013) and -Cu (0.5 - 2.5 nM; Boye et al., 2012). Thus, these earlier studies indicate that the macronutrients (nitrate, phosphate and silicate) as well as dissolved Fe ( $< 0.09\text{nM}$ ) deficiency impose limitation on phytoplankton growth in the STF region (Indian Sector, Sedwick et al., 2002; Blain et al., 2002). However, effect of micronutrients on bacterial growth and

community structure in the Indian Sector of the STF region, are largely unknown. In this regard, experimental addition of 2.5 nM Fe by Church et al (2000) led to 2.5 fold increase in bacterial abundance as well as bacterial production in the water sample from STF region (Australian Sector). On the other hand, Hutchins et al (2001) observed minor iron-mediated changes in bacterial community structure in the subantarctic region.

In this study, we examined the effect of micronutrient amendments such as cobalt (Co), copper (Cu) and iron (Fe) on total Chlorophyll *a* concentration, bacterial abundance, phytoplankton, and bacterial community structure by conducting an on-board microcosm experiment. For this, a bulk sample of water was collected from deep chlorophyll maximum (DCM) depth of ~60m. Further, over 100 randomly chosen pure cultures of bacterial isolates from this depth were brought to the laboratory for biochemical characterization as well as 16S rRNA gene sequencing in order to note the diversity of culturable bacteria.

## **2. Materials and Methods**

### ***2.1 Enumeration of viable populations of heterotrophic bacteria from sampling site***

Seawater nutrient agar (SWNA) medium containing yeast extract 0.3%, peptone 0.5%, agar 1.5% and seawater: distilled water (50:50 V/V) was used to quantify the culturable or ‘platable’ populations of aerobic, heterotrophic bacterial colony forming units (CFU) from different depths at station-1 (stn-1, latitude: 39° 59'0.975S; longitude: 58° 29'0.919E) shown in Fig (1). Briefly, 100 µl of the seawater sample was spread plated onto the SWNA. The plates were incubated at 4°C, 10°C and 20°C until visible bacterial colonies were seen on the plates. The bacterial cultures were purified by streaking single isolated colony using sterile microbiological loop onto the fresh SWNA plates. The plates were wrapped, stored at 4°C, and brought back to the laboratory. Membrane filtration technique was followed so as to not miss out low numbers and to avoid erroneous enumeration due to over-crowding of CFU. For this 2 liters of seawater sample was filtered through 0.2 µm pore size polycarbonate filter (Millipore, USA). After filtration the filter papers were placed on the top of the SWNA plates and incubated at 4°C, 10°C and 20°C until visible bacterial colonies were seen (data not shown).

### ***2.2 Isolation and biochemical characterization of bacterial cultures***

Cultivation-based or culture-dependent approach is important, since ecological role of prokaryotes in natural environments can be assessed only when they are successfully cultivated and characterized.

Sequences of genes cloned directly from environmental DNA are shown to not correspond to the genes of cultured marine taxa (Suzuki et al., 1997). Therefore, we studied cultivable bacterial diversity from stn-1 at the DCM depth (Fig.1) to note the possible diversity in the DCM from where the bulk sample was drawn for onboard microcosm experiments. For this, well isolated and morphologically distinct colonies were selected, purified and brought to the laboratory. Overall, 105 cultures were isolated and 99 of the finally available isolates were subjected to various morphological and biochemical tests (Table-1). Bacterial cultures were clustered in to groups using numerical profiling based on the results of biochemical characteristics. Up to 3 or 4 representative cultures from each group was taken up for identification on the basis of 16S rRNA gene sequencing.

### ***2.3 Extraction of genomic DNA and amplification of 16S rRNA gene from bacterial cultures***

The genomic DNA from the pure cultures was extracted using Genomic DNA isolation kit (Sigma-Aldrich, USA), as per manufacturer's instructions. Bacterial 16S rRNA gene was amplified by following standard Polymerase chain reaction (PCR) method using universal primer set, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACT T-3') developed by Weisburg et al (1991). The PCR mixture (50  $\mu$ l) contained 1 $\mu$ l of extracted DNA (5-50 ng  $\mu$ l<sup>-1</sup>), 1  $\mu$ l of each primer at a concentration of 0.5 $\mu$ M, 25 $\mu$ l of Ready MixTaq PCR mix (Sigma-Aldrich, USA) [1.5 U Taq DNA polymerase; 10mM Tris-HCl, 50mM KCl, 1.5mM MgCl<sub>2</sub>, 0.001% gelatin, 0.2mM deoxynucleoside triphosphate (dNTP), stabilizers], and 22 $\mu$ l of milliQ water. Then the PCR was carried out with the temperature profile as follows. Initial denaturation step of 3 min at 95 °C, followed by 25 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec. The final extension step was for 10 min at 72°C. The PCR was performed via Thermocycler (Applied Biosystems, USA). PCR products thus obtained were checked on 1% agarose gel.

### ***2.4 Sequencing and phylogenetic analysis of culturable bacteria***

The purified PCR products were then cloned into pCR<sup>®</sup>4-TOPO vector (Invitrogen, USA) and transformed into *E. coli* TOP-10 competent cells according to manufacturer's instructions. Colony-PCR was used for checking the positive transformants containing appropriate size of DNA insert, using M13-20 forward and M13 reverse primers (TOPO-TA cloning guide, Invitrogen, USA), which corresponded to both sides of the cloning site on the vector. The clones were then sequenced using ABI 31310XL (Applied Biosystems, USA) genetic analyzer housed in our laboratory. The sequences obtained were edited with the software Vecscreen (<http://www.ncbi.nlm.nih.gov/tools/vecscreen/>), and compared with

the NCBI database through BLAST searches (<http://blast.ncbi.nlm.nih.gov>). In this comparison, sequences of type strains most closely related to the sequences of the isolates were searched. The sequences were aligned with Muscle, and the trees were constructed from the evolutionary distances by the neighbor joining method with the software Mega 5.

### ***2.5 Microcosm experimental setup***

An on-board microcosm experiment was conducted at stn-1 (Fig. 1). For this experiment, 80 liters of seawater was collected from the deep chlorophyll maxima (~ 60 m) depth with a clean Niskin sampler. After passing through 200  $\mu\text{m}$  mesh, the seawater (5 L per flask) was distributed to pre-cleaned flasks of 5 L capacity. Prior to the experiment, the flasks were sequentially cleaned as described in Jain et al (2015 this issue) and were finally rinsed with the sample seawater. ACS grade trace metal salts (Sigma-Aldrich, India) were dissolved in 0.01 N HCl, pH~1.0. After filling with sample seawater, one ml concentrated trace metal solutions viz, Co, Cu, Fe, and Fe + Co + Cu were added to the flasks so as to attain a 10 nM L<sup>-1</sup> final concentration. Some flasks were left unamended and treated as control microcosms (CM). All the flasks were incubated in the shipboard cold room maintained at 16± 2°C. A set of fifteen flasks including 12 micronutrient enriched and 3 unamended control flasks were exposed to a light intensity of 22  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  (under cool fluorescent light) in 16:8h light: dark cycle for 15 days. The samples for bacterial abundance, phytoplankton counts, and bacterial community analysis were collected at 5 day interval. A set of two flasks, one dark control (DC) and another containing mixture of Fe + Co + Cu solutions (Dmix), were incubated under complete dark conditions and were analyzed only after 15 days of incubation to evaluate the direct effect of micronutrient amendment on heterotrophic microbial assemblages.

### ***2.6 Measurements of dissolved nutrients and dissolved organic carbon***

For dissolved nutrient analysis 100 ml sample (in duplicate) from each microcosm was filtered through 0.22  $\mu\text{m}$  pore size membrane filter (Millipore, USA) and stored frozen at -80 °C until analyzed. The nutrients (nitrate, nitrite, silicate, and phosphate) were estimated using Skalar autoanalyzer (SkalarAnalytical, The Netherlands) setup back at NIO, Goa, following standard methods (Grasshoff et al., 1983). For quantifying dissolved organic carbon concentration from each microcosm subsamples of 100ml in duplicate were filtered through pre-ashed GF/F filter paper (Whatman, USA), collected in acid cleaned high density polyethylene bottles, and frozen at -80 °C, until analyzed back in the laboratory.

The dissolved-organic carbon (DOC) was estimated using Liquid TOC analyzer (Liqui TOC II, Elementar HANAU, Germany) as per manufacturer's protocol.

### ***2.7 Total chlorophyll *a*, phytoplankton counts and composition***

For estimating total Chlorophyll *a* 1.5 liter of sample from each flask was filtered through GF/F filter paper (Whatman, USA). The filter paper was stored at -80 °C, until extraction was performed back home in the laboratory. The pigments were extracted under dark conditions in 10 ml of 90% acetone overnight at -20 °C. This extract was then measured in a fluorometer (Turner Design 10AU, USA) to detect chlorophyll *a* fluorescence, using the method described earlier by Welschmeyer (1994). For enumerating total phytoplankton counts and their community structure subsamples of 200 ml were fixed with few drops of acidified Lugol's iodine. The phytoplankton were counted and identified as described earlier by (Naik et al., 2011). In brief, 200ml sample was kept settling for 48 h. After that, the volume was brought down to 10 ml final concentration. From this three replicate of 1 ml each were pipette into sedgewick rafter chamber and examined under an Olympus inverted microscope at ×100 and ×400 magnifications. Identification of the dinoflagellate taxa to generic/species level was carried out using the keys provided by Tomas (1997), and Horner (2002).

### ***2.8 Enumeration of total bacterial cells***

Total bacterial counts (TBC) were made using a modified method of Porter and Feig (1980). In brief, subsamples of 50 ml (in duplicates) were fixed with buffered formaldehyde (2% final concentration) and stored at 4°C in the dark until taken up for analysis. The samples were analyzed within 30 days after reaching NIO, Goa. One to five ml aliquots of these samples were incubated with 4'6'-diamidino-2-phenylindole (DAPI, 20µl of 1µg ml<sup>-1</sup> working solution per ml) and filtered onto black 0.22 µm-pore-size polycarbonate membrane filters (Millipore, U.S.A). Epifluorescence microscopic counts were made using Olympus BX-51 microscope with the help of Image Pro-plus software (Media Cybernetics, USA). TBC were made from a minimum of 20 randomly chosen microscopic fields for each sample to obtain a reliable mean.

### ***2.9 DNA extraction from microcosms***

For extraction of DNA, 2.0 liters of seawater sample from each microcosm was passed at 60 ml min<sup>-1</sup> through a Sterivex cartridge fitted with 0.22 µm pore size membrane filter (Millipore, USA) using a peristaltic pump. Sterivex cartridge was filled with 1.8 ml of lysis buffer (50 mM Tris pH 8.3, 40 mM

EDTA and 0.75 M sucrose), sealed and, stored frozen at -80°C until nucleic acid extraction was done in the laboratory following the modified method of Ferrari and Hollibaugh (1999). After this extraction the DNA was collected and suspended in 40µl TE buffer. Finally, DNA integrity was checked by agarose (0.8 %) gel electrophoresis and the DNA extracts were stored at -80°C until taken up for further analyses.

### ***2.10 PCR amplification***

DNA extracted from all microcosms was subjected to DGGE-PCR, using the method described in Singh and Ramaiah (2011). In brief, V3 region of bacterial 16S rRNA gene was amplified, using DGGE specific primers sets, 357F-GC (5'-GCCCCGCCGCGCGGGCGGGCGGGGCAC

GGGGGGCCCTACGGGAGGCAGAG-3') and 518R (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993), in the thermocycler (Veriti, Applied Biosystems, USA). The PCR mixture (50 µl) contained 1µl of extracted DNA (5-50 ng µl<sup>-1</sup>), 1 µl of each primer at a concentration of 0.5µM, 25µl of ReadyMix Taq PCR mix (Sigma-Aldrich, USA) [1.5 U Taq polymerase; 10mM Tris-HCl, 50mM KCl, 1.5mM MgCl<sub>2</sub>, 0.001% gelatin, 0.2mM deoxynucleoside triphosphate (dNTP), stabilizers], and 22µl of milli Q water. The DGGE-PCR was carried out with the following temperature profile: an initial denaturation step of 3 min at 95°C, followed by 10 cycles of 94°C for 30 sec, 65-55°C for 30 sec (decrease by 1° C after every cycle) and 72°C for 30 sec. Additional 15 cycles were allowed at the 55°C annealing temperature. The final extension step was for 10 min at 72°C. To maximize the detection of as many 'species' as possible, at least three different PCR reactions were carried out for each sample. Replicate PCR products thus obtained were pooled before performing DGGE.

### ***2.11 DGGE analysis***

In order to examine the effect of micronutrient amendments on the microbial community structure, detection of DGGE bands was carried out using the D-Code universal mutation detection system as per the manufacturer's instructions (Bio-Rad, USA). DNA concentration in the PCR products was quantified using Nano-drop (Thermo-Fisher, USA) and equal concentration of DNA per sample (~1.5 µg per lane) was loaded on the DGGE gel. The PCR products were resolved on 10% (w/v) polyacrylamide gel containing 30% to 60% denaturing gradient of urea and formamide for 16 hr at 60 °C at a constant voltage of 60 V in 1X TAE (40 mM tris-HCl, 20 mM acetic acid, 1 mM EDTA). The gels were stained for 30 min in 1X TAE buffer containing ethidium bromide (0.5 µg ml<sup>-1</sup>), washed for

15 min and, visualized in a Gel-Doc system (Bio-Rad, USA) using Quantity One software (Bio-Rad, USA).

### **2.12 Quantitative analysis of DGGE profiles**

Digitized DGGE gel images were analyzed with Quantity-one software (version 4.65, Bio-Rad, USA). Using rolling disk subtraction logic the software carries out a density profile analysis for each lane, detects the bands, and calculates the relative contribution of each band to the total lane intensity. Numbers operational taxonomic units (OTUs) in each sample were counted as number of DGGE bands. The bands occupying the same position in different lanes of the gel were matched with matching algorithm of the software at 1% tolerance level. Gels were cross checked visually as well for number of bands per lane. An intensity matrix was constructed based on the relative contribution of band to the total intensity of the lane. Univariate analyses were performed with PRIMER v6 software package [Plymouth Marine Laboratory, UK]. The total number of DGGE bands was used to calculate the OTU richness ( $S$ ) in each sample. The relative intensity of each band ( $P_i$ ) was used to calculate (a) Shannon diversity index ( $H'$ ) and (b) Pielou's equitability index ( $J'$ ) to describe the possible changes in the dominances among DGGE-OTUs according to the following formulae:

$$H' = -\sum_{i=1}^S P_i(\ln P_i) \quad (a)$$

$$J' = \frac{H'}{\ln S} \quad (b)$$

Where  $S$  is the total number of OTUs and  $P_i$  is the relative intensity of the  $i^{\text{th}}$  OTU.

### **2.13 Excision and sequencing of DGGE bands**

The excision and sequencing of DGGE bands were performed using the modified method of Singh and Ramaiah, (2011). In brief, a total of 12 bands at different position in the gel were excised using a sterile scalpel and re-amplified using primers 357F (without GC clamp) and 518 R. This yielded a fragment/product of 193 bp length. The purified PCR products were then cloned into pCR<sup>®</sup>4-TOPO vector (Invitrogen, USA) and transformed into *E. coli* TOP-10 competent cells according to manufacturer's instructions. The clones were then sequenced using ABI 3130XL genetic analyzer (Applied Biosystems, USA) and their accession numbers obtained from NCBI Genbank. These sequences were compared with NCBI database sequences using BLAST algorithm.



### **3. Results**

#### ***3.1 Abundance of colony forming units and biochemical characteristics***

Though the retrievable CFU were high when grown at 20 °C, there was no discernible trend in their numbers with increasing depth (Table 1). One way analysis of variance (ANOVA) showed that the viable bacterial counts at 20 °C incubation temperature were significantly higher ( $F_{n=15}=12.38$ ;  $P < 0.002$ ) than those growing at lower temperatures.

Biochemical characteristics of as many as 99 pure bacterial cultures (Supplementary table S.1) imply that most of them are capable of growth at 37°C and were positive for oxidase enzyme that catalyzes oxidation-reduction reaction involving molecular oxygen (O<sub>2</sub>) as the electron acceptor. None of the isolates were positive for catalase, nitrate reductase, gelatinase, hydrogen sulphide production, acid production, and indole production (Supplementary table S.1).

#### ***3.2 Phylogenetic analysis of bacterial cultures***

Phylogenetic tree of the isolated bacterial cultures is shown in fig (2). According to the partial 16S rRNA gene sequences, nine major OTU were identified. Of those, seven belonged to *Firmicutes* and two to *Gammaproteobacteria*. In *Firmicutes* group, three were affiliated to genus *Bacillus*, and one each to *Salinicoccus*, *Staphylococcus* and *Pediococcus*. In *Gammaproteobacteria* group, one belonged to *Pseudoalteromonas* sp and the other to *Alteromonas* sp. All nine sequences were non-chimeric and submitted to the NCBI GenBank database. The accession numbers obtained are KJ917333 to KJ917341.

#### ***3.3 Nutrients and dissolved organic carbon concentrations***

The concentration of dissolved silicate increased with the incubation period (Table 2) in all microcosms. Notably, nitrate concentration decreased only in the CM and Co-added microcosms. There was no significant variation in the DOC concentrations between CM and MEM (Table 3). Interestingly, DOC concentration in the dark control (DC) was half of that in the dark-mixture (D-mix).

#### ***3.4 Effect of micronutrient amendments on total Chlorophyll a and phytoplankton abundance***

Total chlorophyll *a* and phytoplankton counts were lower in MEM than that of the CM (Table 4). The total numbers of major phytoplankton groups varied with the incubation period in all the microcosms. However, no significant differences in phytoplankton community structure were observed between CM

and MEM at the end of incubation period, except in dark control (DC) and dark mixture (D-mix) (Table 4). Surprisingly, autotrophic dinoflagellates were found in DC and D-mix at day 15.

### **3.5 Effect of micronutrient amendments on bacterial abundance**

Bacterial abundance in Fe amended microcosms was 3 fold higher than that of CM after 5 days of incubation. Following rapid increase in bacterial abundance within 5 days of incubation, abundance gradually decreased throughout the experiment in the Fe amended microcosms (Fig.3). Unlike in Fe enriched microcosms, the highest bacterial abundance in Control, Co, Cu, and Fe+ Co+ Cu amended microcosms were observed only after 10 days of incubation. Interestingly, bacterial abundance in dark control was 2 fold higher than that of control exposed to the light: dark cycle, after 15 days of incubation. However, bacterial abundance in the dark mixture (D-mix) was similar to that of control, slightly lower than other micronutrient amendments (exposed to the light: dark cycle) and half of the dark control (DC) after 15 days of incubation period.

### **3.6 Effect of micronutrient amendments on bacterial community**

The DGGE banding pattern (Fig.4) and number of OTUs (Table 5), reflect significant change in the bacterial community composition between CM and MEM during the experiment. From the DGGE gel it is evident that the average OTU richness, Shannon diversity index ( $H'$ ) and Pielou's evenness index ( $J'$ ) decreased with the incubation period in control, Co, Fe and mix added microcosms (Table 5), except in Cu amended microcosms wherein the diversity remained similar throughout the incubation. In all 10 different DGGE bands found in the MEM (marked with black arrow head in Fig. 4) were either absent or faintly present in the CM. These phylotypes /DGGE bands vary from eight in Fe to five each in the Co and Cu amended microcosms (Fig.4).

### **3.7 Phylogenetic analysis of DGGE bands**

A total of 12 DGGE bands were excised, cloned and sequenced. Of these, three sequences affiliated to *Gammaproteobacteria*, three to *Cyanobacteria*, two each to *Alphaproteobacteria* and *Bacteroidetes*, and one each to *Firmicutes* and *Betaproteobacteria* (Table 6). Based on DGGE band sequences a phylogenetic tree was constructed to compare their affiliation to the sequences in NCBI database (Fig.5). Most of the sequences matched very well (~99% similarity) with the uncultured marine bacteria reported from tropical ocean (Fig.5, Table 6), except a very few reported from river and soil. The bacterial community in the initial seawater sample and CM was dominated by members of *Gammaproteobacteria*

followed by *Alphaproteobacteria*. In Co enriched microcosm the two most prominent bands B-7 and B-10 were affiliated with *Psychrobacter* sp and *Prochlorococcus* sp, respectively. In the microcosms amended with solution of Cu, Fe, and mixture the most prominent band B-9 was identified as member of family *Cytophagaceae*. The other less intense but unique DGGE bands found in the MEM affiliated with *Cyanobacteria* (B1, B5), *Betaproteobacteria* (B2), *Flavobacteria* (B3), *Roseobacter* (B4), *Firmicutes* (B8) and *Vibrio* sp.(B12) (Table 5). Since all 12 sequences were non-chimeric they were submitted to the GenBank database. The accession numbers obtained for the DGGE bands are KJ872797 to KJ872808.

## **4. Discussion**

### **4.1 Culturable bacterial characteristics**

The higher viable bacterial counts at 20°C compared to the other lower incubation temperatures suggests that the bacteria in the STF region are psychrotrophic, which can grow at low temperature (4°C) but their optimum temperature range is ~20-25°C. More number of strains isolated from STF location aligned to the *Firmicutes* group which mainly comprised of the Gram-positive bacteria. Previous studies have reported a greater presence of Gram-positive bacteria in marine environment (Zhuang et al., 2003; Gontang et al., 2007; Sass et al., 2008), and in several cases these microorganisms are dominant among the cultivable bacteria (Toffin et al., 2004; Gärtner et al., 2011; Velmurugan et al., 2011). Most of the strains identified in *Firmicutes* were related to endospore-forming (EF) *Bacillus*. The ability to produce endospores and the great metabolic and physiological diversity are two characteristics of the EF *Bacillus* that allow their distribution in all environments of our planet (Priest, 1993) and this may explain their presence in our samples.

The second dominant group in our samples was *Gammaproteobacteria* of genera *Alteromonas* and *Pseudoalteromonas*. The bacteria from this phylogenetic group are among the most known and readily cultivable microorganisms from the marine environment (Fuhrman and Hagström, 2008), as noted in this and many other studies (Yanagibayashi et al., 1999; Kobayashi et al., 2008; Ettoumia et al., 2010; Finnegan et al., 2011). The genus *Pseudoalteromonas* has often been found in close association with marine eukaryotic hosts (Holmström et al., 1999) and known for producing agarolytic enzymes involved in the algal tissue degradation (Ivanova et al., 2002). Recently, McCarren et al (2010) indicated the involvement of *Alteromonadales* (specifically, *Idiomarina* spp. and *Alteromonas macleodii*) in

metabolizing semi-labile high molecular weight dissolve organic matter to methanol or formaldehyde, and carbon dioxide.

#### ***4.2 Dissolved nutrient and organic carbon variability in microcosms***

The increase in dissolved silicate concentrations during incubation period in all microcosms might be taken to indicate silica regeneration due to bacterial enzymatic release from dead/decomposing diatoms (Sedwick et al., 2002). Bidle and Azam, (2001) reported that much of the silica regeneration in seawater is due to bacterial enzymatic activity and that diatom decomposition and silica release is highly accelerated in the presence of an active colonizing bacterial population. Similar results were observed from the microcosm experiment we performed during 2013 at a Polar front location (Jain et al., 2015 this issue). The decrease in nitrate concentration in the microcosms might be taken to indicate nitrate reduction. It is very likely that there were alteration in BC in the microcosms leading to non-syntrophic skewing favoring a few nutritionally versatile clades that became apparently dominant and showed up more intensely in our DGGE. At least some of them can be construed to be nitrate reducing. The semi-labile nature of the dissolved organic matter might be responsible for the relatively stable concentration of DOC between CM and MEM, as noted earlier by Smetacek et al (2012) during LOHAFEX experiment.

#### ***4.3 Effect on total Chlorophyll *a*, phytoplankton and bacterial abundance***

Earlier studies reported macronutrients (nitrate, phosphate and silicate) as well as dissolved Fe deficiency impose limitation on phytoplankton growth/total chlorophyll *a* in the STF region (Sedwick et al., 2002; Blain et al., 2002). Therefore, it is likely that lower macronutrient concentration in our sampled STF location might have limited the phytoplankton growth/ total chlorophyll *a*, in spite of our micronutrient addition. The exact reason for the presence of autotrophic dinoflagellates in DC and D-mix is not clear to us. However, in all probability, the sturdy autotrophic cells did not get degraded until after 15 days and it is also possible that they may have been the remnants which did not break off in the dark.

Higher bacterial abundance in Fe amended microcosm after 5 days of incubation might be suggestive of the fact that bacterial growth in the STF region was limited by Fe and they responded rapidly to the Fe availability in the microcosms. It is quite likely that Fe addition directly induced bacterial growth in the microcosm, as reported earlier by Pakulski et al. (1996), Cochlan.(2001), Hutchins et al. (2001) and Jain et al.(2015, this issue).The decrease in bacterial abundance after day 5 in the Fe amended flasks may,

presumably, be due to the depletion of labile organic matter [Becquevort et al., 2007; Jain et al., 2015 (this issue)], and/or reduced availability of Fe with the incubation (Church et al., 2000). The Fe availability favors both bacterial growth efficiency as well as ability to utilize the DOM in the Southern Ocean (Church et al., 2000). Kirchman et al. (2000) reported that growth of heterotrophic bacteria is primarily limited by organic carbon in Fe-poor waters, but when organic carbon limitation is relieved, Fe may rapidly become a limiting factor. Higher bacterial abundance after 15 days of incubation in DC than that of light control not only suggests uncoupling between light driven primary production and organic matter driven bacterial growth but also the ability of heterotrophs to benefit from the leachates due to phytoplankton exudations from senescent, moribund cells, and/or dead phytoplankton and finally by decomposing the cell mass.

The decreased bacterial abundance in the dark incubated mixture amended (D-mix) microcosms compared to the DC might bring forth the fact that under complete dark condition bacteria do decompose/utilize the organic carbon derived from dying phytoplankton faster in the presence of micronutrients. By day 15, their growth may have become micronutrient-limited. We inferred so from the works of Becquevort et al. (2007), who observed increased utilization of dissolved organic matter in presence of high Fe concentration compared to that in low Fe microcosm experiment. In the absence of micronutrients, we believe however, that there is delayed bacterial response to DOC utilization. This might, in all likelihood, have resulted in the increased bacterial abundance in the DC by day 15. This might be the reason for higher DOC accumulation in the D-mix compared to that of the DC by day 15. It is quite likely that the micro fauna (ciliates, heterotrophic dino- and/or nano- flagellates) could also affect the DOC. This needs experimental confirmation.

#### ***4.4 Effect on bacterial community composition***

The dominance of *Gammaproteobacteria* and *Alphaproteobacteria* in the initial sample is similar to that reported earlier from the Southern Ocean (Simon et al., 1999; West et al., 2008; Manganelli et al., 2009; Straza et al., 2010; Ducklow et al., 2011). It is evident from the DGGE profiles that there is enrichment of specific phlotypes in the MEM. This could be either due to successful competition for resources as well as to efficient uptake of bio-available organic carbon (Kirchman and Rich, 1997; Church et al., 2000, Van Wambeke et al., 2002). In this study, the BC composition between CM and MEM are quite different from experiments conducted by Hutchins et al. (2001), Arrieta et al.(2004) and Jain et al. (2015 this issue). Further, Hutchins et al. (2001) reported that the bacterial community composition in the

subarctic Pacific, the Southern Ocean, and the California coastal upwelling region scarcely changed after iron infusions, although their abundance and production increased. Arrieta et al. (2004) also reported relatively stable bacterial community composition inside and outside the iron-enriched patch, during in situ iron-fertilization experiment carried out in the Southern Ocean. However, results from this study are similar to those of Becquevort et al. (2007), Eldridge et al. (2007) and Kataoka et al. (2009). Becquevort et al. (2007) observed significant difference in bacterial community structure between low-Fe and high-Fe amended microcosms. Eldridge et al. (2007) showed that the bacterial community changed in response to iron availability in the Peruvian upwelling region of the equatorial Pacific Ocean. Kataoka et al. (2009) suggested that the responses of heterotrophic bacteria to iron enrichment could differ among phylotypes during an in situ iron-enrichment experiment (SEEDS-II) in the western subarctic Pacific.

#### **4.5 Phylogenetic characterization of DGGE bands from microcosm experiment**

The members of *Cytophaga-Flavobacteria* (CF) cluster represent an important component of the bacterioplankton in the Southern Ocean seawater and sea-ice zones (Abell and Bowman, 2005a, b). In general, members of the CFB phylum can be potentially dominant components of seawater microbial communities either on marine aggregates (DeLong et al., 1993; Rath et al., 1998) and/or in the planktonic phase (Fandino et al., 2001) and are exceptionally responsive to phytoplankton blooms (Pinhassi et al., 2004). The members of CF cluster are chemoorganotrophic, proficient in degrading various biopolymers, particularly the high molecular weight fraction in seawater, including proteins, polysaccharides and diatom debris (Kirchman, 2002), and are known for the algicidal activity (Imai et al., 1993; Sohn et al., 2004). As such, these phylotypes are also known to mediate biogenic silica dissolution (Bidle et al., 2003). Their dominance in MEM, particularly in Cu, Fe and mixture amended microcosms, suggests that in presence of micronutrients they are not only degrading/utilizing the exudations from phytoplankton but also are involved in the algicidal activity and/or biogenic silica regeneration. This partly explains observed decrease in phytoplankton abundance as well as increase in the silicate concentration in the MEM with the incubation period during the microcosm experiment.

Phylogenetic analysis of the DGGE bands also suggests that members of genus *Prochlorococcus* preferentially responded to Co addition. The elemental composition of phytoplankton suggests that Co is an important micronutrient for the growth of cyanobacteria (Sunda and Huntsman, 1995). Cobalt is involved in important biological functions and molecules such as vitamin B12 and carbonic anhydrase

(Morel et al., 1994; Bertrand et al., 2007). Furthermore, *Prochlorococcus* sp are known to have specific requirement of Co (Saito et al., 2002) for their growth and they plays a major role in removing dissolved Co from the euphotic zone (Shelley et al., 2012).

Other unique but less intense phylotypes in MEM belongs to the *Psychrobacter* sp, *Roseobacter* sp, *Bacillus* sp, *Burkholderia* sp, and *Vibrio* sp. Members of genus *Psychrobacter* are chemoheterotrophic and cold adapted. However, omics' technologies have revealed that these bacteria harbor adaptive traits that could promote their dispersion beyond low temperature locations (Methe et al., 2005; Cacace et al., 2010). They play fundamental role in biogeochemical cycles and are important biotechnologically for cold adapted enzymes production (Feller & Gerday, 2003). The *Roseobacter* clade is reported to preferentially use phytoplankton products (Moran et al., 2002; Grossart et al., 2005; Howard et al., 2006) and are often reported during Iron enrichment experiments (Kataoka et al., 2009; Thiele et al., 2012). The members of *Bacillus* genus are often reported from terrestrial environment. However, Liu et al. (2013) showed that *Bacillus* of marine origin is phylogenetically well separated from those of terrestrial origin. Maliji et al. (2013) reported that members of *Bacillus* are involved in the hydrocarbons degradation in marine ecosystem.

## 5. Conclusions

Our experiments are useful to recognize rapid response of bacteria to iron enrichment and weaker if at all response from the phytoplankton. This could be taken to indicate that bacterial communities are iron limited. Observed higher bacterial abundance in dark incubation suggests both weak bacterial-phytoplankton coupling in the STF and the ability of heterotrophs to benefit from death and decaying phytoplankton. Variations, both in abundance and BC, might be implying selective shift in the bacterial community composition due to preferential and/or versatile utilization of exogenously added micronutrients.

## Acknowledgements

We thank Director CSIR-NIO for the support and encouragement. Financial support from CSIR funded project PSC0206 is gratefully acknowledged. We thank, Chief Scientist of SOE-2012 Dr. C.T. Achuthankutty for his excellent onboard support. We appreciate Dr. Anil Kumar (Coordinator-Indian expedition to Southern Ocean) for the logistic-support. We also thank Dr. Basha Shaik (Senior Scientist, CSIR-CSMCRI, Bhavnagar) for analyzing DOC samples. Excellent and very detailed review advisories by two anonymous experts did help immensely in improving this manuscript. This is NIO contribution number 5731.

## References

- Abell, G.C.J., Bowman, J.P., 2005a. Colonization and community dynamics of class Flavobacteria on diatom detritus in experimental mesocosms based on Southern Ocean seawater. *FEMS. Microbiol. Ecol.* 53, 379-391.
- Abell, G.C.J., Bowman, J.P., 2005b. Ecological and biogeographic relationships of class *Flavobacteriain* the Southern Ocean. *FEMS. Microbiol. Ecol.* 51, 265-277.
- Arrieta, J.M., Weinbauer, M.G., Lute, C., Herndl, G.J., 2004. Response of bacterioplankton to iron fertilization in the Southern Ocean. *Limnol.Oceanograph.*49 (3), 799-808.
- Becquevort, S., Lancelot, C., Schoemann, V., 2007. The role of iron in the bacterial degradation of organic matter derived from *Phaeocystis Antarctica*. *Biogeochem.*83,119-135.
- Belkin, I.M., 1988. Main hydrological features of the central south Pacific, in: Vinogradov, M.E., Flint, M.V. (Eds.), *English Translation, Pacific Subantarctic Ecosystems.*, N. Z. Translation Centre Wellington, pp. 12-17.
- Bertrand, E.M., Saito, M.A., Rose, J.M., Riesselman, C.R., Lohan, M.C., Noble, A.E., Lee, P.A., DiTullio, G.R., 2007. Vitamin B-12 and iron colimitation of phytoplankton growth in the Ross Sea. *Limnol. Oceanograph.* 52, 1079-1093.
- Bidle, K.D., and Azam, F., 2001. Bacterial control of silicon regeneration from diatom detritus: Significance of bacterial ectohydrolases and species identity. *Limnol. Oceanogr.* 46, 1606-1623.
- Bidle, K.D., Brzezinski, M.A., Long, R.A., Jones, J.L., Azam, F., 2003. Diminished efficiency in the oceanic silica pump caused by bacteria-mediated silica dissolution. *Limnol. Oceanogr.* 48, 1855-1868.
- Blain, S., Sedwick, P.N., Griffiths, F.B., Queguiner, B., Bucciarelli, E., Fiala, M., Pondaven, P., Treguer, P., 2002. Quantification of algal iron requirements in the Subantarctic Southern Ocean (Indian sector). *Deep-Sea Res. II* 49, 3255-3273.
- Boyd, P., LaRoche, J., Gall, M., Frew, R., McKay, R.M.L., 1999. Role of iron, light, and silicate in controlling algal biomass in subantarctic waters SE of New Zealand. *J. Geophys. Res.* 104, 13391-13404.
- Boye, M., Wake, B.D., Lopez Garcia, P., Bown, J., Baker, A. R., Achterberg, E.P., 2012. Distributions of dissolved trace metals (Cd, Cu, Mn, Pb, Ag) in the southeastern Atlantic and the Southern Ocean. *Biogeosciences.* 9(8), 3231-3246.
- Cacace, G., Mazzeo, M.F., Sorrentino, A., Spada, V., Malorni, A., Sicilian, R.A., 2010. Proteomics for the elucidation of cold adaptation mechanisms in *Listeria monocytogenes*. *J. Proteomics* 73, 2021-2030.
- Castrillejo, M., Statham, P.J., Fones, G.R., Planquette, H., Idrus, F., Roberts, K., 2013. Dissolved trace metals (Ni, Zn, Co, Cd, Pb, Al, and Mn) around the Crozet Islands, Southern Ocean. *J. Geophysical. Res: Oceans.* 118, 1–14.



- Church, M.J., Hutchins, D.A., Ducklow, H.W., 2000. Limitation of bacterial growth by dissolved organic matter and iron in the Southern Ocean. *Appl. Environ. Microbiol.* 66, 455- 466.
- Coale, K.H., Johnson, K.S., Fitzwater, S.E., Gordon, R.M., et al, 1996. A massive bloom induced by an ecosystem-scale iron fertilization experiment in the equatorial Pacific Ocean. *Nature*. 383, 495-500.
- Cochlan, W.P. 2001. The heterotrophic bacterial response during a mesoscale iron enrichment experiment (IronEx II) in the eastern equatorial Pacific Ocean. *Limnol.Oceanogr.* 46, 428-435.
- Coppola, L., Roy-Barman, M., Mulsow, S., Povinec, P., Jeandel, C., 2005. Low particulate organic carbon export in the frontal zone of the Southern Ocean (Indian sector) revealed by <sup>234</sup>Th. *Deep-Sea Res. I* 52, 51-68.
- Croot, P.L., Baars, O., Streu, P. 2011. The distribution of zinc in the Atlantic sector of the Southern Ocean. *Deep-Sea Res. II* 58, 2707-2719.
- Currie, K., Reid, M., Hunter, K., 2011. Interannual variability of carbon dioxide drawdown by subantarctic surface water near New Zealand. *Biogeochem.* 104, 23-34.
- Currie, K.I., Hunter, K.A., 1998. Surface water carbon dioxide in the waters associated with the subtropical convergence, east of New Zealand. *Deep-Sea Res. I* 45, 1765-1777.
- deBaar, H.J.W., Boyd, P.W., Coale, K.H., Landry, M.R., Tsuda, A., Assmy, P., Bakker, D.C.E., et al., 2005. Synthesis of iron fertilization experiments: From the Iron Age in the Age of Enlightenment. *J Geophys Res: C* 110, C09S16.
- DeLong, E.F., Franks, D.G. and Alldredge, A.L. 1993. Phylogenetic diversity of aggregate attached versus free-living marine bacterial assemblages. *Limnol. Oceanogr.* 38, 924-934.
- Ducklow, H.W., Myers, K.M.S., Erickson, M., Ghiglione, J.F., Murray, A.E., 2011. Response of a summertime Antarctic marine bacterial community to glucose and ammonium enrichment. *Aquat. Microb. Ecol.* 64, 205-220.
- Eldridge, M.L., Cadotte, M.W., Rozmus, A.E., Wilhelm, S.W., 2007. The response of bacterial groups to changes in available iron in the Eastern subtropical Pacific Ocean. *J. Exp. Mar. Biol. Ecol.* 348, 11-22.
- Ellwood, M.J., 2004. Zinc and cadmium speciation in subantarctic waters east of New Zealand. *Mar. Chem.* 87, 37-58.
- Ellwood, M.J., vandenBerg, C.M.G., Boye, M., Veldhuis, M., Jong, J.T.M., deBaar, H.J.W., de Croot, P.L., Kattner, G., 2005. Organic complexation of cobalt across the Antarctic Polar Front. *Mar. Freshwater. Res.* 56, 1069-1075.
- Ellwood, M.J., 2008. Winter time trace metal (Zn, Cu, Ni, Cd, Pb and Co) and nutrient distributions in the subantarctic zone between 40– 52°S; 155–160°E. *Mar. Chem.* 112, 107-117.

- Ettoumia, B., Bouhajjaa, E., Borinb, S., Daffonchiob, D., Boudabousa, A., Cherif, A., 2010. Gammaproteobacteria occurrence and microdiversity in Tyrrhenian Sea sediments as revealed by cultivation-dependent and -independent approaches. *Syst. Appl. Microbiol.* 33, 222-231
- Fandino, L.B., Riemann, L., Steward, G.F., Long, R.A., Azam, F., 2001. Variations in bacterial community structure during a dinoflagellate bloom analysed by DGGE and 16S rDNA sequencing. *Aquat. Microb. Ecol.* 23, 119-130.
- Feller, G., Gerday, C., 2003. Psychrophilic enzymes: hot topics in cold adaptation. *Nat. Rev. Microbiol.* 1, 200-208.
- Ferrari, V.C., Hollibaugh, J.T., 1999. Distribution of microbial assemblages in the Central Arctic Ocean Basin studied by PCR/DGGE: analysis of a large data set. *Hydrobiologia.* 401, 55-68.
- Finnegan, L., Garcia-Melgares, M., Gmerek, T., Huddleston, W.R., Palmer, A., Robertson, A., Shapiro, S., Unkles, S.E., 2011. A survey of culturable aerobic and anaerobic marine bacteria in de novo biofilm formation on natural substrates in St. Andrews Bay, Scotland. *Antonie Leeuwenhoek.* 100, 399-404.
- Fuhrman, J.A., Hagstrom, A., 2008. Bacterial and archaeal community structure and its patterns. In: Kirchman, DL (eds). *Microbial Ecology of the Oceans*, 2nd edn. John Wiley & Sons, Inc., Hoboken, NJ, USA. pp 45–90.
- Gärtner, A., Blümel, M., Wiese, J., Imhoff, J.F., 2011. Isolation and characterization of bacteria from the Eastern Mediterranean deep sea. *Antonie. Leeuwenhoek.* 100, 421-435.
- Gontang, E.A., Fenical, W., Jensen, P.R., 2007. Phylogenetic diversity of Gram-positive bacteria cultured from marine sediments. *Appl. Environ. Microbiol.* 73, 3272-3282.
- Grasshoff, K., Ehrhardt, E., Kremling, K., 1983. *Methods of Seawater Analysis*, 2<sup>nd</sup> ed., Verlag Chemie, Weinheim, p 401
- Grossart, H.P., Levold, F., Allgaier, M., Simon, M., Brinkhoff, T., 2005. Marine diatom species harbour distinct bacterial communities. *Environ. Microbiol.* 7 (6), 860-873.
- Hassler, C.S., Sinoir, M., Clementson, L.A., Butler, E.C.V., 2012. Exploring the link between micronutrients and phytoplankton in the Southern Ocean during the 2007 austral summer. *Front. Microbiol.* 3, 1.
- Holmstrom, C., Kjelleberg, S., 1999. Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiol. Ecol.* 30, 285-293
- Howard, E.C., Henriksen, J.R., Buchan, A., Reisch, C.R., Buergmann, H., Welsh, R., Ye, W.Y., Gonzalez, J.M., Mace, K., Joye, S.B., Kiene, R.P., Whitman, W.B., Moran, M.A., 2006. Bacterial taxa that limit sulfur flux from the ocean. *Science.* 314 (5799), 649-652
- Horner, R.A., 2002. *A taxonomic guide to some common marine phytoplankton*, Bristol, England, UK, Biopress, Bristol, pp. 1–195.

- Hutchins, D.A., Campbell, B.J., Cottrell, M.T., Takedaand, S., Cary, S.C., 2001. Response of marine bacterial community composition to iron additions in three iron-limited. *Limnol. Oceanogr.* 46, 1535-1545.
- Imai, I., Ishida, Y., Hata, Y., 1993. Killing of marine phytoplankton by a gliding bacterium *Cytophaga* sp., isolated from the coastal Sea of Japan. *Mar. Biol.* 116: 527-532.
- Ito, T., Woloszyn, M., Mazloff, M., 2010. Anthropogenic carbon dioxide transport in the Southern Ocean driven by Ekman flow. *Nature* 463, 80-83.
- Ivanova, E.P., Sawabe, T., Alexeeva, Y.V., Lysenko, A.M., Gorshkova, N.M., Hayashi, K., Zukova, N.V., Christen, R., Mikhailov, V.V., 2002. *Pseudoalteromonas sissachenkonii* sp. nov., a bacterium that degrades the thallus of the brown alga *Fucusevanescens*. *Int. J. Syst. Evol. Microbiol.* 52:229-234.
- Jain, A., Bandekar, M., Gomes, J., Meena, R.M., Ramaiah, N., 2015. Response of Polar front phytoplankton and bacterial community to micronutrient amendments. *Deep Sea Research. II* (this issue).
- Jasmine, P., Muraleedharan, K.R., Madhu1, N.V., Asha Devi1, C.R., Alagarsamy, R., Achuthankutty, C.T., Jayan, Z., Sanjeevan, V.N., Sahayak, S., 2009. Hydrographic and productivity characteristics along 45°E longitude in the southwestern Indian Ocean and Southern Ocean during austral summer 2004. *Mar. Ecol. Prog. Ser.* 389, 97-116.
- Jayne, S.R., Marotzke, J., 2002. The oceanic eddy heat transport. *J. Phys. Oceanogr.* 32, 3328-3345.
- Kataoka, T., Suzuki, K., Hayakawa, M., Kudo, I., Higashi, S., Tsuda, A., 2009. Temporal changes in community composition of heterotrophic bacteria during in situ iron enrichment in the western subarctic Pacific (SEEDS-II). *Deep-Sea. Res. II* 56, 2779-2787.
- Kirchman, D.L., Rich, J., 1997. Regulation of bacterial growth rates by dissolved organic carbon and temperature in the equatorial Pacific Ocean. *Microb. Ecol.* 33, 11-20.
- Kirchman, D.L., Meon, B., Cottrell, M.T., Hutchins, D.A., Weeks, D., Bruland, K.W., 2000. Carbon versus Iron Limitation of Bacterial Growth in the California Upwelling Regime. *Limnol. Oceanogr.* 45(8), 1681-1688.
- Kirchman, D.L., 2002. The ecology of *Cytophaga*-Flavobacteria in aquatic environments. *FEMS. Microbiol. Ecol.* 39, 91-100.
- Kobayashi, T., Koide, O., Mori, K., Shimamura, S., Matsuura, T., Miura, T., Takaki, Y., Morono, Y., Nunoura, T., Imachi, H., Inagaki, F., Takai, K., Horikoshi, K., 2008. Phylogenetic and enzymatic diversity of deep seafloor aerobic microorganisms in organics- and methane-rich sediments off Shimokita Peninsula. *Extremophiles* 12 (4), 519-527.
- Kostianoy, A.G., Ginzburg, A.I., Frankignoulle, M., Delille, B., 2004. Fronts in the southern Indian Ocean as inferred from satellite sea surface temperature data. *J. Mar. Syst.* 45, 55-73.

- Liu, Y., Lai, Q., Dong, C., Sun, F., Wang, L., Li, G., Shao, Z., 2013. Phylogenetic diversity of the *Bacillus pumilus* group and the marine ecotype revealed by multilocus sequence analysis. *Plos. One.* 8: e80097.
- Manganelli, M., Malfatti, F., Samo, T.J., Mitchell, B.G., Wang, H., Azam, F., 2009. Major Role of Microbes in Carbon Fluxes during Austral Winter in the Southern Drake Passage. *PLoS. One.* 4, 6941.
- Maliji, D., Olama, Z., H, Holail., 2013. Environmental studies on the microbial degradation of oil hydrocarbons and its application in Lebanese oil polluted coastal and marine ecosystem. *Int. J. Curr. Microbiol. App. Sci.* 2, 1-18.
- McCarren, J., Becker, J.W., Repeta, D.J., Shi, Y., Young, C.R., Malmstrom, R.R., Chisholm, S.W., DeLong, E.F., 2010. Microbial community transcriptomes reveal microbes and metabolic pathways associated with dissolved organic matter turnover in the sea. *PNAS* 107, 16420-16427.
- Methe, B.A., Nelson, K.E., Deming et al., 2005. The psychrophilic lifestyle as revealed by the genome sequence of *Colwelliapsychrerythraea* 34H through genomic and proteomic analyses. *P. Natl. Acad. Sci. USA.* 102, 10913-10918.
- Metzl, N., Tilbrook, B., Poisson, A., 1999. The annual fCO<sub>2</sub> cycle and the air-sea CO<sub>2</sub> flux in the sub-Antarctic Ocean. *Tellus B* 51, 849-861.
- Metzl, N., 2009. Decadal increase of oceanic carbon dioxide in Southern Indian Ocean surface waters (1991-2007). *Deep-Sea Res. II* 56, 607-619.
- Moran, X.A.G., Estrada, M., Gasol, J.M., PedrosAlio, C., 2002. Dissolved primary production and the strength of phytoplankton bacterioplankton coupling in contrasting marine regions. *Microb. Ecol.* 44 (3), 217-223.
- Morel, F.M.M., Reinfelder, J.R., Roberts, S.B., Chamberlain, C.P., Lee, J.G., Yee, D., 1994. Zinc and carbon co-limitation of marine-phytoplankton. *Nature* 369, 740-742.
- Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59, 695-700.
- Naik, R.K., Hegde, S. Anil, A.C., 2011. Dinoflagellate community structure from the stratified environment of the Bay of Bengal, with special emphasis on harmful algal bloom species *Environ. Monit. Assess.* 182, 15-30.
- Orsi, A.H., Whitworth III, T., Nowlin Jr., W.D., 1995. On the meridional extent and fronts of the Antarctic circumpolar current. *Deep-Sea Res. I* 42, 641-673.
- Pakulski, J.D., Coffin, R.B., Kelley, C.A., Holder, S.L., Downer, R., Aas, P., Lyons, M. M., Jeffrey, W.H., 1996. Iron stimulation of Antarctic bacteria. *Nature.* 383, 133-134.
- Porter, K.G., Feig, Y.S., 1980. The use of DAPI for identifying and counting aquatic microfloral. *Limnol. Oceanogr.* 25, 943-948.

- Rath, J., Wu, K.Y., Hernd, G.J., DeLong, E.F., 1998. High phylogenetic diversity in a marine snow associated bacterial assemblage. *Aquat. Microb. Ecol.* 14, 261-269.
- Robinson, C., Ramaiah, N., 2011. Microbial heterotrophic metabolic rates constrain the microbial carbon pump, in: *Microbial carbon pump in the ocean*, The American Association for the Advancement of Science, USA, pp. 52-53.
- Pakhomov, E.A., McQuaid, C.D., 1996. Distribution of surface zooplankton and seabirds across the Southern Ocean. *Polar. Biol.* 16, 271-286.
- Pinhassi, J., Sala, M.M., Havskum, H., Fransesc, P., Guadayol, O., Malits, A., Marrase, C., 2004. Changes in bacterioplankton composition under different phytoplankton regimes. *Appl. Environ. Microbiol.* 70, 6753-6766.
- Priest, F.G., 1993. Systematics and ecology of *Bacillus*. In *Bacillus subtilis and Other Gram-positive Bacteria*, pp. 3–33. Edited by Sonenshein, A.L., Hoch, J.A., Losick, R., Washington, DC: American Society for Microbiology.
- Saito, M.A., Moffett, J.W., Chisholm, S.W., Waterbury, J.B., 2002. Cobalt limitation and uptake in *Prochlorococcus*. *Limnol. Oceanogr.* 47(6), 1629-1636.
- Sass, A.M., Mckew, B.A., Sass, H., Fichtel, J., Timmis, K.N., Mcgenity, T.J., 2008. Diversity of *Bacillus*-like organisms isolated from deep-sea hypersaline anoxic sediments. *Saline. Syst.* 4, 8-18.
- Sedwick, P.N., Blain, S., Queguiner, B., Griffiths, F.B., Fiala, M., Bucciarelli, E., Denis, M., 2002. Resource limitation of phytoplankton growth in the Crozet Basin, Subantarctic Southern Ocean. *Deep-Sea Res. II* 49, 3327-3349.
- Shelley, R. U., Sedwick, P. N., Bibby, T. S., Cabedo-Sanz, P., Church, T. M., Johnson, R. J., Macey, A. I., Marsay, C. M., Sholkovitz, E. R., Ussher, S. J., Worsfold, P. J., Lohan, M. C., 2012. Controls on dissolved cobalt in surface waters of the Sargasso Sea: Comparisons with iron and aluminum. *Global Biogeochem. Cy.* 26, GB2020, doi:10.1029/2011GB004155.
- Shadwick, E.H., Trull, T.W., Tilbrook, B., Sutton, A.J., Schulz, E., Sabine, C.L., 2015. Seasonality of biological and physical controls on surface ocean CO<sub>2</sub> from hourly observations at the Southern Ocean Time Series site south of Australia. (In press) DOI: 10.1002/2014GB004906.
- Singh, S.K., Ramaiah, N., 2011. Denaturing gradient gel electrophoresis profiling of bacterial communities composition in Arabian Sea. *J. Environ. Biol.* 32, 339-346.
- Simon, M., Glöckner, F.O., Amann, R., 1999. Different community structure and temperature optima of heterotrophic picoplankton in various regions of the Southern Ocean. *Aquat. Microb. Ecol.* 18, 275-284.
- Smetacek, V., Klaas, C., Strass, V.H., Assmy, P., Montresor, M., Cisewski, B., Savoye, N., et al 2012. Deep carbon export from a Southern Ocean iron-fertilized diatom bloom. *Nature.* 487, 313-319.
- Sohn, J.H., Lee, J.H., Yi, H., Chun, J., Bae, K.S., Ahn, T.Y., Kim, S.J., 2004. *Kordiaalgicida* gen. nov., sp. nov., an algicidal bacterium isolated from red tide. *Int. J. Syst. Evol. Microbiol.* 54, 675-80.

- Straza, T.R.A., Ducklow, H.W., Murray, A.E., Kirchman, D.L., 2010. Abundance and single-cell activity of bacterial groups in Antarctic coastal waters. *Limnol.Oceanogr.* 55, 2526-2536.
- Sunda, W.G., Huntsman, S.A., 1995. Cobalt and zinc interreplacement in marine phytoplankton: biological and geochemical implications. *Limnol.Oceanogr* 40, 1404-1417.
- Suzuki, M.T., Rappé, M.S., Haimberger, Z.W., Winfield, H., Adair, N., Ströbel, J., Giovannoni, S.J., 1997. Bacteria diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. *Appl. Environ. Microbiol.* 63, 983-989.
- Thiele, S., Fuchs, B.M., Ramaiah, N., Amann, R., 2012. Microbial Community Response during the Iron Fertilization Experiment LOHAFEX. *Appl. Environ. Microbiol.* 78, 8803-8812.
- Tomas, C. R. 1997. Identifying marine phytoplankton. San Diego, California: Academic. pp. 387-589.
- Toffin, L., Webster, G., Weightman, A.J., Fry, J.C., Prieur, D., 2004. Molecular monitoring of culturable bacteria from deep-sea sediment of the Nankai Trough, Leg 190 Ocean Drilling Program. *FEMS.Microbiol. Ecol.* 48, 357-367.
- Van Wambeke, F., Christaki, U., Giannakourou, A., Moutin, T., Souvemerzoglou, K., 2002. Longitudinal and vertical trends of bacterial limitation by phosphorus and carbon in the Mediterranean Sea. *Microb. Ecol.* 43, 119-133.
- Velmurugan, N., Kalpanaa, D., Chob, J.Y., Leec, G.H., Park, S.H., Leea, Y.S., 2011. Phylogenetic analysis of culturable marine bacteria in sediments from South Korean Yellow Sea. *Microbiol.* 80, 261-272.
- West, N.J., Obernosterer, I., Zemb, O., Lebaron, P., 2008. Major differences of bacterial diversity and activity inside and outside of a natural iron-fertilized phytoplankton bloom in the Southern Ocean. *Environ. Microbiol.* 10, 738-756.
- Welschmeyer, N.A. 1994. Fluorometric analysis of chlorophyll a in the presence of chlorophyll b and pheopigments. *Limnol.Oceanogr.* 39(8), 1985-1992
- Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J., 1991. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol.* 173(2); 697-703.
- Yanagibayashi M, Nogi Y, Li L, Kato C (1999) Changes in the microbial community in Japan Trench sediment from a depth of 6292 m during cultivation without decompression. *FEMS MicrobiolLett* 170:271-279
- Zhuang, W.Q., Tay, J.H., Maszenan, A.M., Krumholz, L.R., Tay, S.T.L., 2003. Importance of Gram-positive naphthalene-degrading bacteria in oil-contaminated tropical marine sediments. *Lett. Appl. Microbiol.* 36, 251-257.

## Tables

Table1

Viable bacterial counts from samples collected at surface, DCM, 200, 500, and 1000m from stn-1, incubated at three different temperatures.

Depth	Viable counts x 10 <sup>3</sup> ml <sup>-1</sup> at different incubation temperature		
	4-6 °C	10°C	20°C
Surface	0.05	0.022	4.3
DCM	0.022	0.005	0.8
200m	0.052	0.03	3
500m	0.590	0.355	5.7
1000m	0.065	0.027	7.8

Table 2

Macronutrient concentrations in control microcosm (CM) and micronutrient enriched microcosm (MEM) during the experimental period.

Treatment	Nutrient ( $\mu\text{mol/L}$ )	Incubation time (days)			
		Day-0	Day-5	Day-10	Day-15
Control	Nitrite	$0.70 \pm 0.023$	$0.69 \pm 0.035$	$1.24 \pm 0.09$	$0.89 \pm 0.084$
	Nitrate	$1.50 \pm 0.099$	$1.43 \pm 0.1$	$0.62 \pm 0.058$	$0.07 \pm 0.0025$
	Phosphate	$0.56 \pm 0.051$	$0.64 \pm 0.045$	$0.89 \pm 0.065$	$1.06 \pm 0.1$
	Silicate	$1.89 \pm 0.12$	$2.47 \pm 0.15$	$7.24 \pm 0.21$	$8.74 \pm 0.32$
Cobalt	Nitrite		$0.8 \pm 0.052$	$1.01 \pm 0.086$	$1.14 \pm 0.094$
	Nitrate		$2.5 \pm 0.12$	$1.92 \pm 0.19$	$0.54 \pm 0.053$
	Phosphate		$0.6 \pm 0.049$	$0.73 \pm 0.068$	$1.09 \pm 0.093$
	Silicate		$2.1 \pm 0.164$	$5.32 \pm 0.20$	$8.61 \pm 0.42$
Copper	Nitrite		$1 \pm 0.085$	$1.27 \pm 0.99$	$0.91 \pm 0.74$
	Nitrate		$0.05 \pm 0.001$	$0.07 \pm 0.002$	$1.91 \pm 0.084$
	Phosphate		$0.55 \pm 0.05$	$0.61 \pm 0.045$	$0.95 \pm 0.67$
	Silicate		$2.05 \pm 0.153$	$5.17 \pm 0.179$	$7.90 \pm 0.198$
Iron	Nitrite		$0.55 \pm 0.036$	$0.71 \pm 0.069$	$0.75 \pm 0.042$
	Nitrate		$0.039 \pm 0.002$	$0.61 \pm 0.025$	$1.28 \pm 0.085$
	Phosphate		$0.51 \pm 0.036$	$0.57 \pm 0.041$	$0.73 \pm 0.052$
	Silicate		$1.98 \pm 0.13$	$6.05 \pm 0.23$	$7.33 \pm 0.31$
Mixture	Nitrite		$0.72 \pm 0.056$	$0.78 \pm 0.38$	$1.13 \pm 0.99$
	Nitrate		$0.55 \pm 0.065$	$1.91 \pm 0.15$	$0.72 \pm 0.065$
	Phosphate		$0.49 \pm 0.032$	$0.50 \pm 0.036$	$0.88 \pm 0.045$
	Silicate		$2.25 \pm 0.14$	$3.98 \pm 0.123$	$7.34 \pm 0.23$
Dark control	Nitrite		-	-	$1.11 \pm 0.158$
	Nitrate		-	-	$1.51 \pm 0.129$
	Phosphate		-	-	$1.11 \pm 0.98$
	Silicate		-	-	$8.18 \pm 0.31$
Dark Mixture	Nitrite		-	-	$1.07 \pm 0.26$
	Nitrate		-	-	$1.05 \pm 0.185$
	Phosphate		-	-	$0.74 \pm 0.085$
	Silicate		-	-	$6.18 \pm 0.345$



Table 3

Dissolved organic carbon ( $\mu\text{M}$ ) concentration in control microcosm (CM) and micronutrient enriched microcosm (MEM) over the incubation period.

	<b>Dissolved organic carbon (DOC, <math>\mu\text{M}</math>)</b>				
	<b>Day-0</b>	<b>Day-5</b>	<b>Day-10</b>	<b>Day-15</b>	<b>Dark-day-15</b>
Control	$26.0 \pm 0.85$	$26.48 \pm 0.75$	$26.56 \pm 0.63$	$30.86 \pm 0.89$	$18.17 \pm 0.22$
Co		$31.12 \pm 0.42$	$27.30 \pm 0.36$	$27.22 \pm 0.34$	
Cu		$27.98 \pm 0.59$	$27.84 \pm 0.69$	$32.87 \pm 0.87$	
Fe		$27.31 \pm 0.64$	$26.19 \pm 0.74$	$25.73 \pm 0.62$	
Mix		$26.44 \pm 0.98$	$23.2 \pm 0.99$	$24.86 \pm 0.87$	$26.6 \pm 0.86$

Table 4

Total Chlorophyll a ( $\mu\text{g L}^{-1}$ ) and total counts per liter of major phytoplankton groups identified in the microcosm experiment.

Treatment	Incubation period	Total chl a ( $\mu\text{g L}^{-1}$ )	Centeric diatoms	Pennate Diatom	Autotrophic dinoflagellates	Mixotrophic dinoflagellates	Heterotrophic dinoflagellates
Control	Day-0	0.07	3015	0	737	134	737
	Day 5	0.09	11000	3900	1500	300	0
	Day 10	0.23	9400	80400	200	0	100
	Day 15	0.08	17000	20700	100	0	0
Cobalt	Day 5	0.06	5000	1700	600	200	1300
	Day 10	0.058	9700	3000	700	100	0
	Day 15	0.045	3800	3000	400	0	200
Copper	Day 5	0.04	2400	1300	1800	300	200
	Day 10	0.035	400	700	500	100	0
	Day 15	0.15	13700	43500	100	0	0
Iron	Day 5	0.019	500	1300	200	0	0
	Day 10	0.024	1600	500	0	0	0
	Day 15	0.029	700	1500	0	0	0
Mixture	Day 5	0.039	400	100	500	100	1300
	Day 10	0.035	200	200	400	0	600
	Day 15	0.002	200	200	0	0	0
Dark	Dark-Control	-	100	0	200	0	400
	Dark-Mixture	-	0	0	400	0	500

Table 5  
 DGGE band based details of OTU richness ( $S$ ), Shannon diversity index ( $H'$ ) and Pielou's evenness index ( $J'$ ) of bacterial community in different microcosms.

Parameters	Amendments	Incubation time (days)				
		Day-0	Day-5	Day-10	Day-15	Dark-day-15
$S$	Control	4	4	3	2	2
	Co		4	1	3	-
	Cu		5	5	5	-
	Fe		9	7	6	-
	Mix		3	2	1	2
$H'$	Control	1.172	1.172	0.568	0.536	1.097
	Co		1.139	0	0.855	-
	Cu		1.531	1.577	1.430	-
	Fe		1.706	1.614	1.126	-
	Mix		1.066	0.656	0	0.679
$J'$	Control	0.845	0.845	0.819	0.774	0.998
	Co		0.821	0	0.778	-
	Cu		0.951	0.980	0.888	-
	Fe		0.876	0.900	0.812	-
	Mix		0.970	0.946	0	0.980

Table 6 Sequence analyses of DGGE bands. Note that ‘B’ stands for the DGGE band.

<b>Band no.</b>	<b>Accession no.</b>	<b>Phylogenetic affiliation</b>	<b>Closest relative</b>	<b>Sim (%)</b>	<b>Environmental description</b>
B1	KJ872797	<i>Cyanobacteria</i>	<i>Prochlorococcus</i> sp. SCGC AAA300-M11 (HQ675336.1)	99	Seawater from Hawaii Ocean Time-Series station
B2	KJ872798	<i>Betaproteobacteria</i>	uncultured <i>Burkholderia</i> sp. clone YN01 (KJ162573.1)	99	Chinese Songhua river mud
B3	KJ872799	<i>Bacteriodetes</i>	uncultured <i>Flavobacterium</i> sp. clone OS3BD113 (JN233025.1)	99	ocean water collected from the continental slope off Cape Lookout North Carolina
B4	KJ872800	<i>Alphaproteobacteria</i>	uncultured <i>Roseobacter</i> sp. clone C114500429 (JX527100.1)	100	Derived from the Southern ocean iron fertilization experiment (LOHAFEX)
B5	KJ872801	<i>Cyanobacteria</i>	uncultured <i>Cyanobacterium</i> clone WS08_021 (KJ566267.1)	99	Seawater from Arctic fjord of Svalbard
B6	KJ872802	<i>Gammaproteobacteria</i>	uncultured <i>Gammaproteobacterium</i> clone K4S173 (JN177694.1)	99	Subsurface seawater from Arctic Ocean
B7	KJ872803	<i>Gammaproteobacteria</i>	<i>Psychrobacter</i> sp. SC35 (EU939718.1)	99	Tidal flat sediment Republic of Korea
B8	KJ872804	<i>Firmicutes</i>	<i>Bacillus pumilus</i> strain H4.9/8 (KJ722435.1)	100	Gross Schoenebeck geothermal station
B9	KJ872805	<i>Cytophagia</i>	uncultured <i>Cytophagaceae</i> bacterium clone CNY_01316 (JQ401295.1)	98	Soil sample from Moab, Utah
B10	KJ872806	<i>Cyanobacteria</i>	uncultured <i>Prochlorococcus</i> sp. clone ASTSB3 (KF724064.1)	99	Seawater from Arabian Sea Time Series station
B11	KJ872807	<i>Alphaproteobacteria</i>	uncultured <i>Alphaproteobacterium</i> clone SHAS476 (GQ349381.1)	99	Seawater from Saanich Inlet
B12	KJ872808	<i>Gammaproteobacteria</i>	<i>Vibrio rumoiensis</i> strain EHP3 (DQ530286.1)	98	luminescent symbionts of Cephalopoda

## Figures

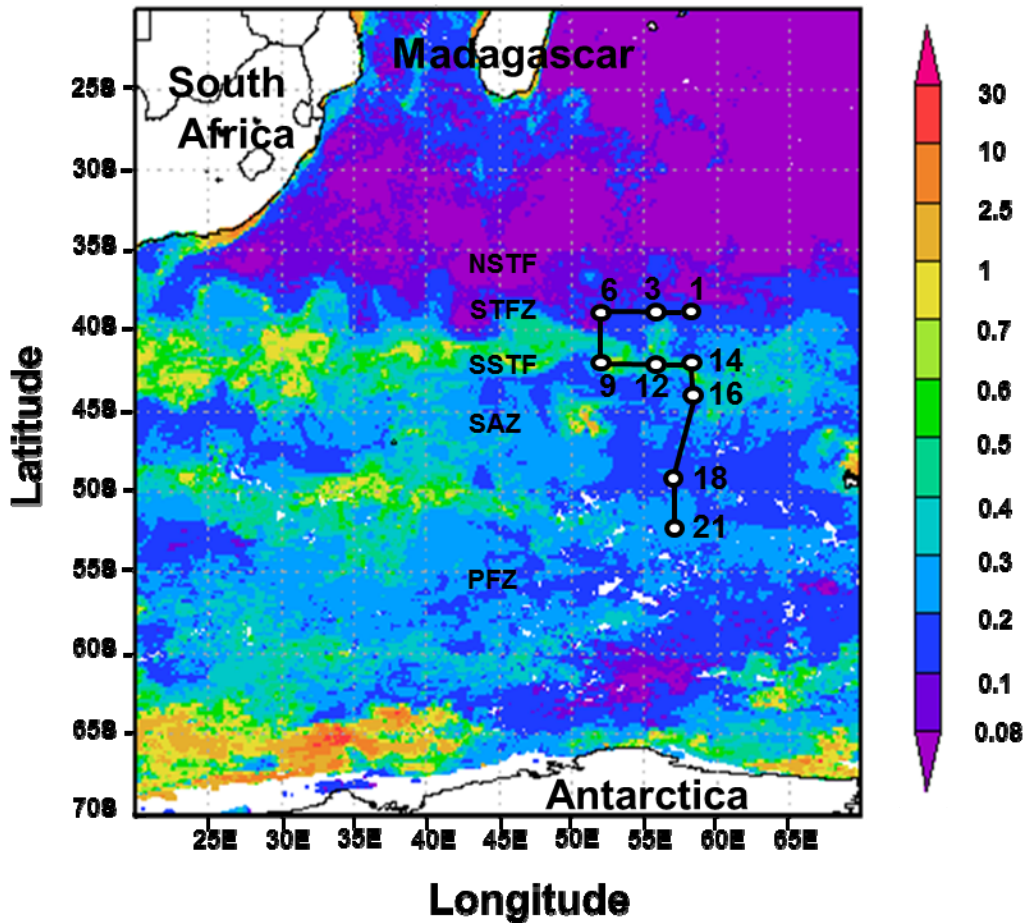


Fig.1. Map showing station location (white circle), overlaid on MODIS-aqua Chl *a* distribution in Indian sector of Southern Ocean (ISSO) during January 2012. Samples for microcosm experiment were collected from Stn-1 (White circle), during the Sixth Indian Expedition to Southern Ocean (SOE-2012). The fronts and frontal zones at 45 °E longitude are marked from Anilkumar et al (2005, 2006, and 2007). NSTF, North subtropical front; STFZ, Subtropical frontal zone; SSTF, South subtropical front; SAZ, Sub-antarctic zone; PFZ, Polar frontal zone.

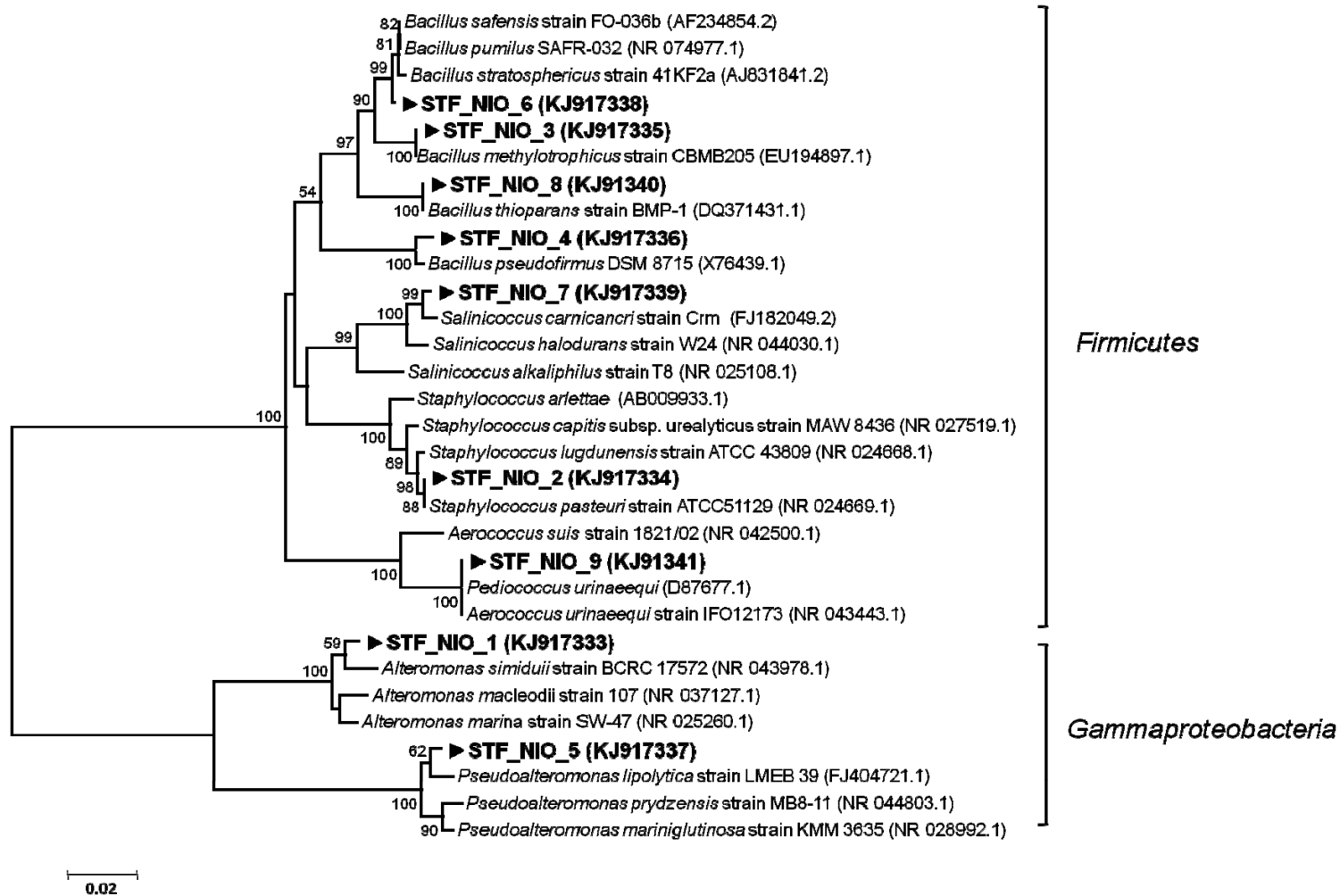


Fig.2. Neighbor-joining tree showing the phylogenetic relationships of 16S rRNA gene sequences of bacterial isolates. Note that bootstrap support values over 50% are shown. The accession number obtained for each strain is shown in the bracket (right side of the strain number). The scale bar indicates evolutionary distance.

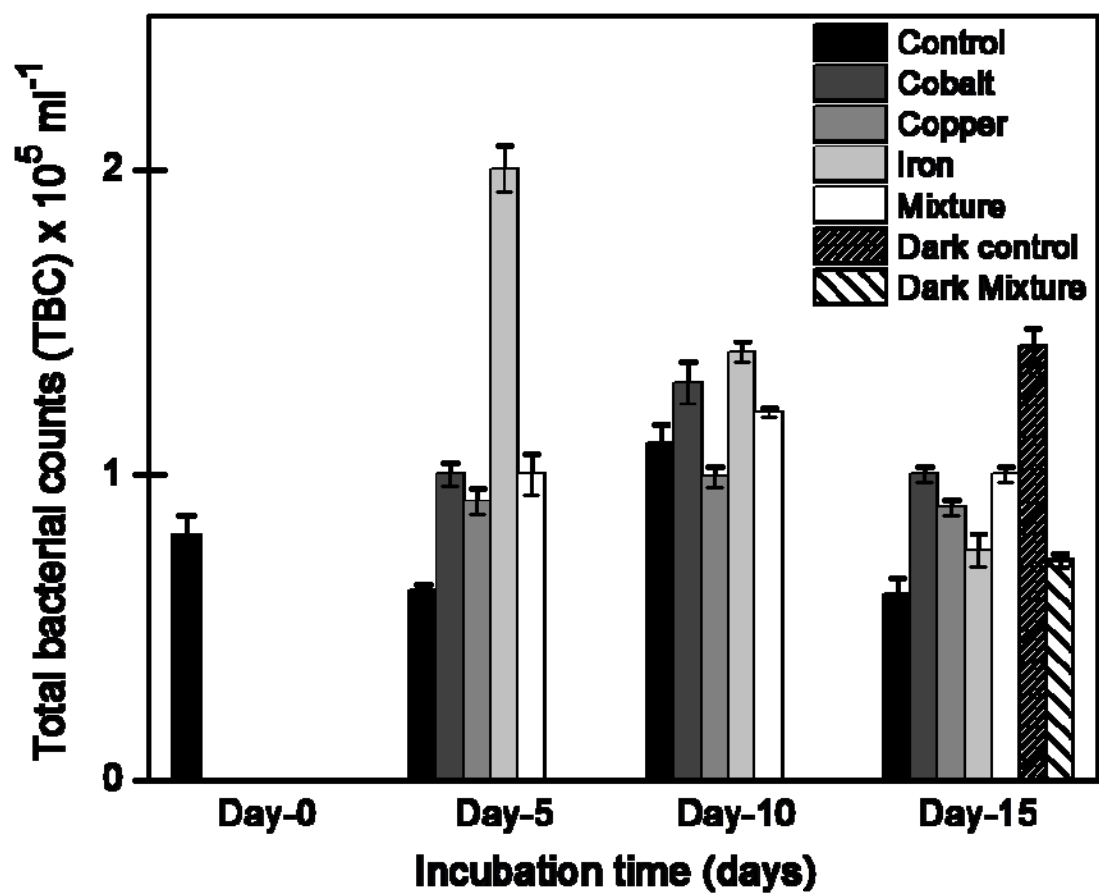


Fig.3. Variations in total bacterial counts over a period of 15 days in the different microcosms.

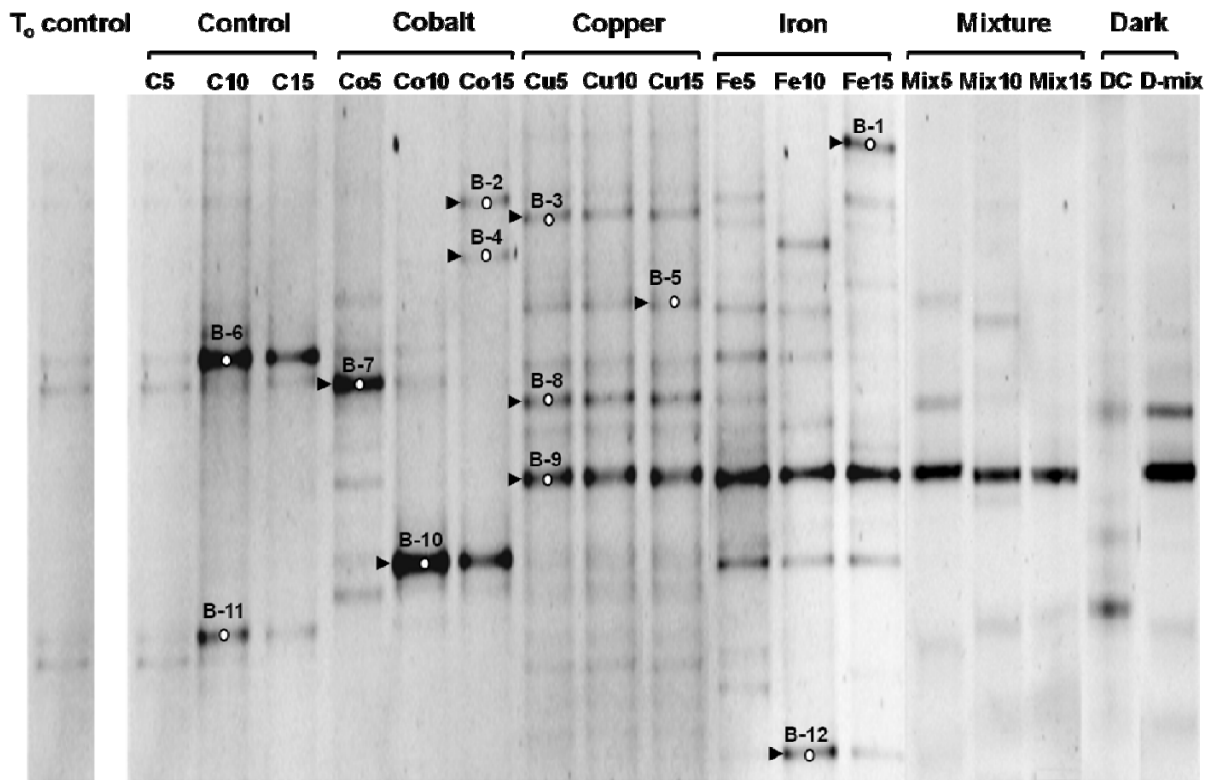


Fig.4. DGGE gel depicting bacterial community of in control microcosms (CM) and micronutrient enriched microcosms (MEM) over the incubation period. The excised bands are shown by a circle on them. The phlotypes/bands in MEM either absent or faintly present in the CM are marked with black arrow head.



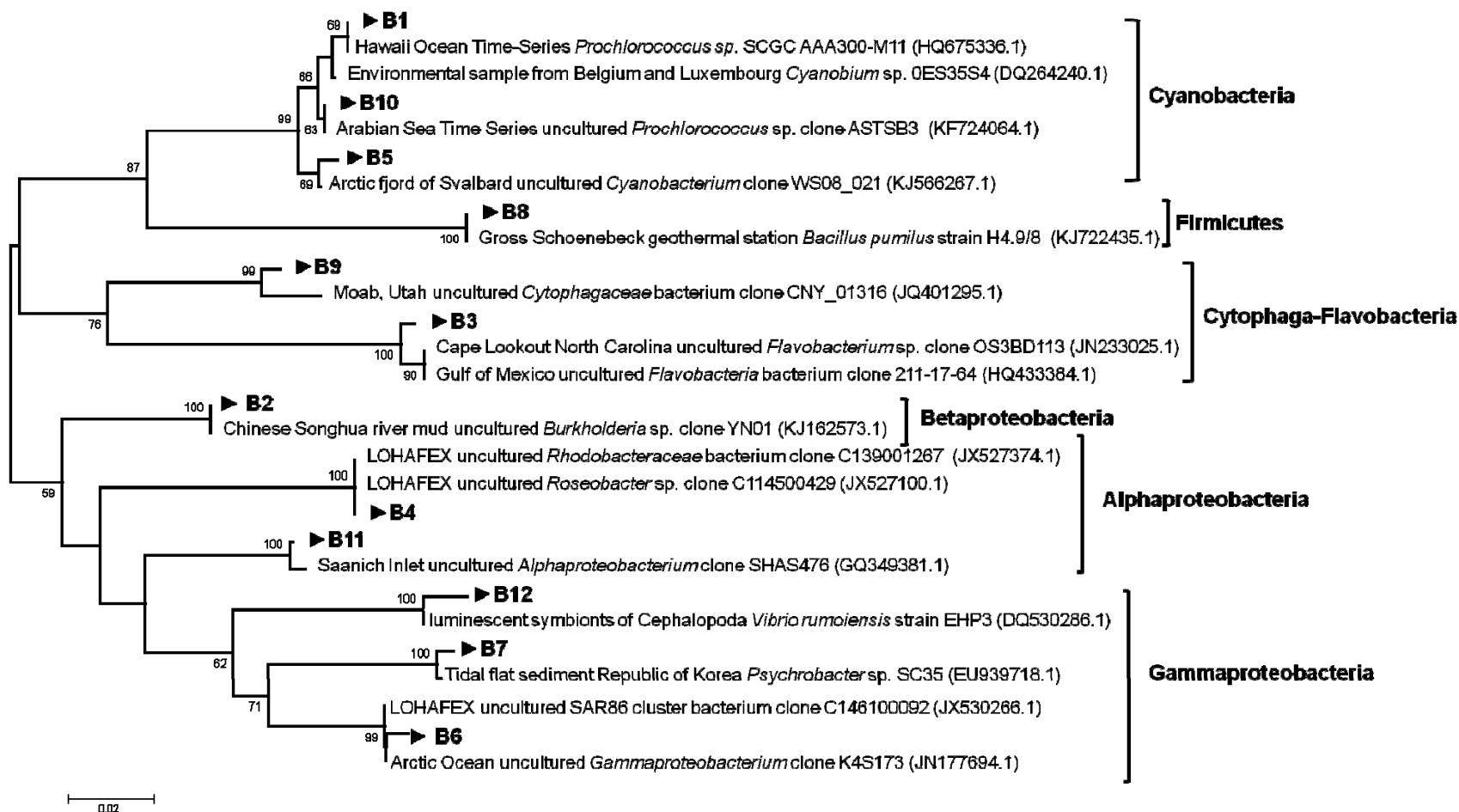


Fig.5. Phylogenetic tree (maximum likelihood) of V3 region of bacterial 16S rRNA gene amplified using DGGE specific primers, based on their affiliation with NCBI database sequences. Bootstrap values (calculated with the neighbour-joining method) > 50% are given at the nodes. The scale bar indicates the percentage of the sequence divergence.

## Supplementary material

Table S.1

Motility and biochemical characteristics of bacterial cultures isolated from the subtropical front.

Isolate no.	Biochemical tests*																			
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	P	Q	R	S	U	
1	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	
2	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	
3	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	
4	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	
5	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	
6	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	
7	+	-	+	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	+	
8	+	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	
11	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	+	
12	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
13	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	
14	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	
15	+	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	+	
16	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	
17	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	
18	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	
19	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	
20	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	
21	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	
22	+	-	+	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-	+	
23	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	
24	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	
25	-	-	+	-	-	+	-	-	-	-	-	-	+	+	-	-	+	-	+	
26	-	-	+	-	-	-	-	+	-	-	-	-	+	+	-	-	-	-	+	
30	+	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	+	
31	+	-	+	-	-	+	-	+	-	-	-	-	+	+	-	-	-	-	+	
32	+	-	+	-	+	+	-	-	-	-	-	-	-	+	-	+	-	-	+	
33	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	
34	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	-	+	-	+	
35	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	+	-	-	+	
36	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	
37	+	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	-	+	+	
38	+	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-	+	
39	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	-	-	-	
41	+	-	+	-	-	-	-	+	-	-	-	-	-	+	-	+	-	+	-	
42	+	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	+	+	-	
43	+	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	-	+	+	
44	+	-	+	-	+	+	-	-	-	-	-	-	-	+	-	+	-	+	+	

45	-	-	+	-	+	-	-	+	-	-	-	-	+	+	-	+	-	+	+
46	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	+	-	+	+
47	+	-	+	-	+	+	-	+	-	-	-	-	-	-	-	+	-	-	+
48	+	-	+	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	+
49	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+
50	+	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	-	-	+
51	+	-	+	-	-	-	-	+	-	-	-	-	-	+	-	+	-	-	+
52	-	-	+	-	+	+	-	+	-	-	-	-	-	+	-	+	-	-	+
67	+	-	+	-	+	+	-	+	-	-	-	-	-	-	-	+	-	-	+
69	+	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-	+
71	+	-	+	-	+	-	-	+	-	-	-	-	-	+	-	+	-	-	+
72	-	-	+	-	+	+	-	-	-	-	-	-	-	+	-	+	+	-	+
73	-	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	+	-	+
74	+	-	+	-	-	+	-	+	-	-	-	-	-	-	-	+	-	-	+
75	+	-	+	-	-	+	-	+	-	-	-	-	+	-	-	+	-	-	+
76	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+
77	+	-	+	-	-	+	-	+	-	-	-	-	+	-	-	+	+	-	+
78	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+
79	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+
80	+	-	+	-	-	-	-	+	-	-	-	-	+	-	-	+	-	-	+
81	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-
82	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+
83	+	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+
84	+	-	+	-	-	+	-	+	-	-	-	-	+	-	-	+	-	-	+
85	-	-	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+
86	+	-	+	-	-	+	-	-	-	-	-	-	+	+	-	+	-	-	+
87	+	-	+	-	-	+	-	+	-	-	-	-	-	+	-	+	-	-	+
88	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+
89	+	-	+	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	+
90	+	-	+	-	+	-	-	-	-	-	-	-	-	+	-	+	+	-	+
91	+	-	+	-	+	-	-	+	-	-	-	-	-	+	-	+	+	-	+
92	+	-	+	-	+	+	-	-	-	-	-	-	+	-	-	+	-	-	+
93	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+
94	+	-	-	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	-
95	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
96	+	-	+	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+
97	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+	-	-	+
98	+	-	+	-	+	-	-	+	-	-	-	-	-	+	-	+	-	-	+
99	+	-	+	-	+	+	-	+	-	-	-	-	-	+	-	+	-	-	+

A-Motility; B- Catalase; C-Oxidase; D-Nitrate Reductase; E-Urease; F-Gelatinase; G-lipase; H-Citrate; I-H<sub>2</sub>S production; J-Methyl Red; K-VogesProskauer; L-Indole; M-Amylase; N-Glucose; P-Rhamnose; Q-Mannitol; R-Maltose; S- Triple sugar iron medium; U-Growth at 37 °C